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Sadeghinezhad, J ; Amrein, I

Abstract: Background: Oxaliplatin (OX) has been widely used for treatment of colorectal and other cancers. Adverse effect of OX and other anticancer agents on cognition have been reported, but studies on the effects of chemotherapy on brain structure are scarce. This study describes the morphometrical features of the hippocampus structures in rat following OX treatment using design-based stereological methods. Materials and methods: Ten male Wistar rats were randomized into two groups. The rats from OX group received 2.4 mg/kg OX in vehicle for five consecutive days every week for 2 weeks intraperitoneally (IP). Controls received vehicle only. Cavalieri 's method and the optical fractionator method were used for volume and neuron estimation, respectively. Results: Cavalieri 's method was used for to estimate volume and showed that the volume of the hippocampus was significantly decreased in OX group (31.84 ± 1.24 mm3) compared with the vehicle control group (36.95 ± 3.48 mm3). The optical fractionator method was used to estimate neuron number and showed that the number of neurons in dentate gyrus, cornu ammonis 1 and 3 in OX group (8.147 ± 2.84 × 105, 4.257 ± 0.59 × 105 and 2.133 ± 0.22 × 105, respectively) did not differ from those of vehicle control group (7.36 ± 1.42 × 105, 3.521 ± 0.54 × 105 and 1.989 ± 0.46 × 105, respectively). Conclusions: These findings suggested that OX treatment induce loss of hippocampal volume without neuronal loss which might help to clarify the mechanism by which OX affects cognition and to improve preventive treatment strategies.

DOI: https://doi.org/10.5603/fm.a2020.0031

Originally published at:
Sadeghinezhad, J; Amrein, I (2021). Stereological analysis of hippocampus in rat treated with chemotherapeutic agent oxaliplatin. Folia Morphologica, 80(1):26-32.
DOI: https://doi.org/10.5603/fm.a2020.0031

ONLINE FIRST

ISSN: 0015-5659

e-ISSN: 1644-3284

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Authors: J. Sadeghinezhad, I. Amrein

DOI: 10.5603/FM.a2020.0031

Article type: ORIGINAL ARTICLES

Submitted: 2019-12-12

Accepted: 2020-01-27

Published online: 2020-03-11

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Running title: Hippocampus after oxaliplatin treatment

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Abstract

Background: Oxaliplatin (OX) has been widely used for treatment of colorectal and other cancers. Adverse effect of OX and other anticancer agents on cognition have been reported, but studies on the effects of chemotherapy on brain structure are scarce. This study describes the morphometrical features of the hippocampus structures in rat following OX treatment using design-based stereological methods.

Materials and methods: Ten male Wistar rats were randomized into two groups. The rats from OX group received 2.4 mg/kg OX in vehicle for five consecutive days every week for 2 weeks intraperitoneally (IP). Controls received vehicle only. Cavalieri 's method and the optical fractionator method were used for volume and neuron estimation, respectively.

Results: Cavalieri 's method was used for to estimate volume and showed that the

volume of the hippocampus was significantly decreased in OX group (31.84 ± 1.24 mm^3) compared with the vehicle control group (36.95 ± 3.48 mm^3). The optical fractionator method was used to estimate neuron number and showed that the number of neurons in dentate gyrus, cornu ammonis 1 and 3 in OX group (8.147 ± 2.84 × 10^5, 4.257 ± 0.59 × 10^5 and 2.133 ± 0.22 × 10^5, respectively) did not differ from those of vehicle control group (7.36 ± 1.42 × 10^5, 3.521 ± 0.54 × 10^5 and 1.989 ± 0.46 × 10^5, respectively).

Conclusions: These findings suggested that OX treatment induce loss of hippocampal volume without neuronal loss which might help to clarify the mechanism by which OX affects cognition and to improve preventive treatment strategies.

Key words: stereology, hippocampus, oxaliplatin, chemotherapy, rat

Introduction

Chemotherapy in many cancer types have increased survival times in patients. However, cognitive dysfunctions, referred to as "chemobrain", such as impairments in attention and concentration, verbal and visual memory and processing speed have been reported after systemic chemotherapy [7,41]. There are numerous reports regarding adverse effect of different anticancer agents like methotrexate [29,37,42,46], 5-fluorouracil [8,11], cyclophosphamide [9,36], doxorubicin [23,27], paclitaxel [19], vincristine [5,35] and cytosine arabinoside [26] on cognition in human and animal experimental models. Chemotherapy agents, which inhibit tumor cell proliferation, can also effect on non-tumor cell proliferation in the brain [7].

Oxaliplatin (OX) is a third-generation platinum drug which has been widely used alone or with other chemotherapeutic agents for treatment of colorectal cancer and other carcinomas including ovarian, breast and lung cancers [34,40]. It is able to react with DNA to create DNA intrastrand adducts, which block DNA synthesis and induce apoptosis in cancer cells and rapidly dividing cells [43]. OX induces peripheral neuropathy [38], crosses the blood-brain barrier and accumulates in cerebrospinal and extracellular fluid in the brain [17,18], which indicates that the drug can have a direct effect on brain function and structure. Treatment with OX has been shown to induce

cognitive impairment in laboratory animals [9,10,39]. Furthermore, cellular damage in the hippocampus has been related to the loss of memory function in rats following OX administration [4,7]. Other platinum compounds such as cisplatin, have also been reported to produce cognitive dysfunction and central neurological problems [28,48].

The hippocampus, as the key structure in learning and the formation of memory is an appropriate site for investigating the mechanisms involved in some of the cognitive problems arisen by the chemotherapeutic agents [4].

Designed-base stereology enables unbiased and precise quantitative analysis of three-dimensional structures [15]. It is one of the important techniques for the morphometrical evaluation of the hippocampus, which contain important information about the memory function [14]. The morphometrical features of the hippocampus following chemotherapy have not been thoroughly investigated using stereology. Assessment of the effect of OX on hippocampus volume using Cavalieri 's method and neuronal number in different regions of the hippocampus using optical disector/fractionator, as a gold standard for efficient, unbiased number estimation in neuroscience, may contribute to evaluate the risks of the treatment and possibly help improve preventive strategies.

MATERIALS AND METHODS

Animals- Ten adult (12-15 weeks) male Wistar rats (Pasteur Institute, Tehran, Iran) were used for the experiment. Animals were housed in large acrylic cages with free access to food and water under controlled light (12 h light and dark cycle) and temperature (22 ±2 °C). The rats were allowed to acclimatize for one week before experimentation.

All experimental procedures involving animals in this study were conducted in accordance with the standard guide for the care and use of laboratory animals of the University of Tehran, Tehran, Iran.

Drug administration- Rats were randomly divided into OX-treated and vehicle control groups. The rats from OX group received 2.4 mg/kg OX dissolved in 5% glucose solution (vehicle) for five consecutive days per week for 2 weeks intraperitoneally (IP) and the vehicle control was administrated 5% glucose solution.

The chosen dose of OX was in accordance with that commonly used for neurotoxic evaluation of the agent in previous animal studies [4].

Tissue sampling and stereological methods- Rats were euthanized 21 days after treatment using thiopental (50 mg/kg IP). Then, transcardial perfusion fixation of the animals was done using 10 % formalin. The brains were removed and the left hemisphere of each animal was fixed in same fixative, dehydrated and embedded in glycol methacrylate (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim/Ts, Germany) according to the manufacturer's instructions.

Embedded brains were cut frontally using a rotary microtome with 20 micrometer thickness. Every 20th section was collected using the principle of systematic uniform random sampling, which is known as the section sampling fraction (ssf= 1/20). On average, this sampling scheme provided 13 (11-15) sections per animal. Sections were mounted, dried and stained in Giemsa solution [16].

Hippocampal volume and principal cell numbers were estimated using StereoInvestigator 10 software (MBF Bioscience, Williston, VT, USA). The hippocampus including dentate gyrus (DG) and cornu ammonis (CA) were determined in the sections using rat brain atlas of Paxinos and Watson (1986). DG were recognized due to horseshoe-shape with small and densely packed neurons. The CA2 region in this study was considered as belong to the CA3 region because the CA2 is very small and the boundaries between these two regions are not detectable. The border between the CA3 and CA1 is defined by a small transition zone between the two regions [21] (Fig. 1).

Estimation of the volume- Estimation of total volume of the hippocampus was done using test points on each section (Fig. 2a). The volume was estimated by Cavalieri estimator using the following equation [15]: $\mathbf{V= \Sigma P \cdot ssf \cdot T \cdot (a/p)}$
where ΣP is the total number of points hitting the structure; ssf (1/20) is the section sampling fraction; T (20 μm) is the section thickness and a/p ($9 \cdot 10^4$ μm^2) represents the area per point.

Estimation of the number of neurons- The optical fractionator was used for number estimation of pyramidal cells and granular cells in CA and DG, respectively. First, the contours of the dentate gyrus and the CA1 and CA3 regions were delineated at low magnification. Then, selected regions were analyzed by systematic random

sampling at high magnification. A known fraction of the each section was assessed by moving the microscope motorized stage in regular step length in x and y directions and applying an unbiased counting frame sized to count the neurons of specific region (Fig. 2b).

For DG, a step length of 180 μm and counting frame size of 12 × 12 μm was used, for CA1 a step length of 240 μm and frame size of 20 × 20 μm, and for CA3 a step length of 190 μm and frame size of 24 × 24 was applied. The counting frame was focused through 10 μm of the section thickness, which corresponds to disector height. Section thickness was measured at every 4-5 sampling location.

The total number of cells in the granular cells (DG) and pyramidal cells (CA1 and CA3) in the left hemisphere was calculated using the following formula [6,32]:

$N = 1/SSF \cdot 1/ASF \cdot 1/HSF \cdot \sum Q^-$

where $HSF = h / tQ^-$

for $tQ^- = \sum t_i q^-_i / \sum q^-_i$

$\sum Q^-$: total count of particles sampled; SSF: the section sample fraction; ASF: the area sample fraction (frame size/ x,y step length); Hsf : the height sampling fraction; h: the dissector height (10 μm in this study); tQ^-: the number-weighted mean section thickness; t_i: the section thickness in the ith counting frame with a cell count of q^-_i in the disector.

Statistical analysis- The coefficient of error (CE) of the volume and the number estimates was calculated as previously described [13]. T-test was used for statistical analysis. A value of $p < 0.05$ was considered significant.

RESULTS

The results showed that the volume of the hippocampus was significantly decreased after OX treatment compared with the vehicle control group ($p < 0.05$). The related volume of hippocampus in OX-treated and vehicle control group was 31.84 ± 1.24 mm^3 and 36.95 ± 3.48 mm^3, respectively (Table 1). The number of neurons in DG, CA1 and CA3 did not show any significant difference between the two groups. The neuronal number of DG, CA1 and CA3 in the OX-treated group was estimated to be 8.147 ± 2.84 × 10^5, 4.257 ± 0.59 × 10^5 and 2.133 ± 0.22 × 10^5 respectively, while the related neurons in vehicle control group accounted 7.36 ± 1.42 × 10^5, 3.521 ± 0.54 ×

10^5 and $1.989 \pm 0.46 \times 10^5$, respectively (Table 1). The CE^2/CV^2 ratios (Table 1) are typically > 0.5 indicating that group variances are dominated by the inter-animal differences with only minor contributions of measurement variance to the group variance.

DISCUSSION

The hippocampus is pivotal for learning and the formation of memory in the brain. Since the hippocampus shows various forms of plasticity, including the formation of new nerve cells in adult animals, it may be affected by the chemotherapeutic drugs leading to cognitive deficit [25].

In the present study, stereological techniques were used for the quantification of volume and number of neurons of the hippocampus following OX treatment in rat. The results of the current experiment demonstrated that treatment with OX caused a reduction in total volume of the hippocampus. Considering that oxaliplatin penetrates the blood brain barrier and accumulates in the brain [17,18], an effect on the hippocampus is plausible and may explain memory impairment and other cognitive dysfunctions that have been found in previous studies using behavioral tests [9,10,39].

Similar to our results, magnetic resonance imaging (MRI) studies showed a reduced hippocampus volume in breast cancer survivors exposed to chemotherapy [1,3,22]. In contrast, Yoshikawa et al. [47], found no adverse effects of adjuvant chemotherapy on hippocampal volume in Japanese breast cancer survivors using MRI. They implied that brain regions other than the hippocampus, such as the prefrontal cortex, might be involved in memory impairment after chemotherapy.

The neuron numbers did not show any significant loss of either granular cells of the DG or pyramidal cells of the CA1 and CA3 following OX treatment in rat. OX has been reported to induce apoptotic pathway in hippocampus with increasing caspase-3 and caspase-9 [4]. Cyclophosphamide chemotherapy has been suggested to suppress hippocampal neurogenesis and interrupt hippocampal function in mice [45]. Methotrexate also showed inhibition of the formation of immature neurons in the hippocampus [12,44]. It is interesting that although hippocampal neuronal loss has been explained well in Alzheimer diseases, Joelving et al. [20] did not find any significant

loss of neuron in Parkinson's disease. Korbo et al. [24] also reported no loss of hippocampal neurons in non-Alzheimer dementia.

It should be mentioned that reduction in the number of neurons is not the only mechanism that affect neuronal function. Structurally, decreasing dendritic branching or losing synapses may impair neuronal function without neuronal loss [1]. Changes in hippocampal transmitters and signal transduction pathways are other mechanisms that may disturb hippocampal function [30,31]. These findings suggest that cognitive disorders may be associated with a variety of changes that may not necessarily include a change in neuronal cell number [20].

It is important to note that the estimating neuron number based on 2-D counting is potentially biased. In our study, the potential bias was avoided by the use of the optical fractionator, which is a combination of fractionator sampling and the optical disector principle. This unbiased stereological method is not influenced by tissue deformation like shrinkage or swelling. Importantly, it is also unaffected by changes in the shape or size of the cells that are being counted. The optical fractionator is the gold-standard method for estimating number of neurons [14,24].

This study is the first description of an effect of oxaliplatin on the rat hippocampus using stereology. In conclusion, the results of the present study showed that OX treatment induces a decrease of hippocampal volume without neuronal loss. These findings might help to clarify the mechanism by which OX affects cognition by crossing the blood-brain barrier and accumulating in cerebrospinal and extracellular fluid in the brain and promote the development of treatment strategies that minimize cognitive side effects.

Acknowledgments

The authors would like to thank Prof. David Wolfer and Dr. Lutz Slomianka for their excellent scientific collaboration.

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Table 1. Estimated hippocampal volume and neuron number.

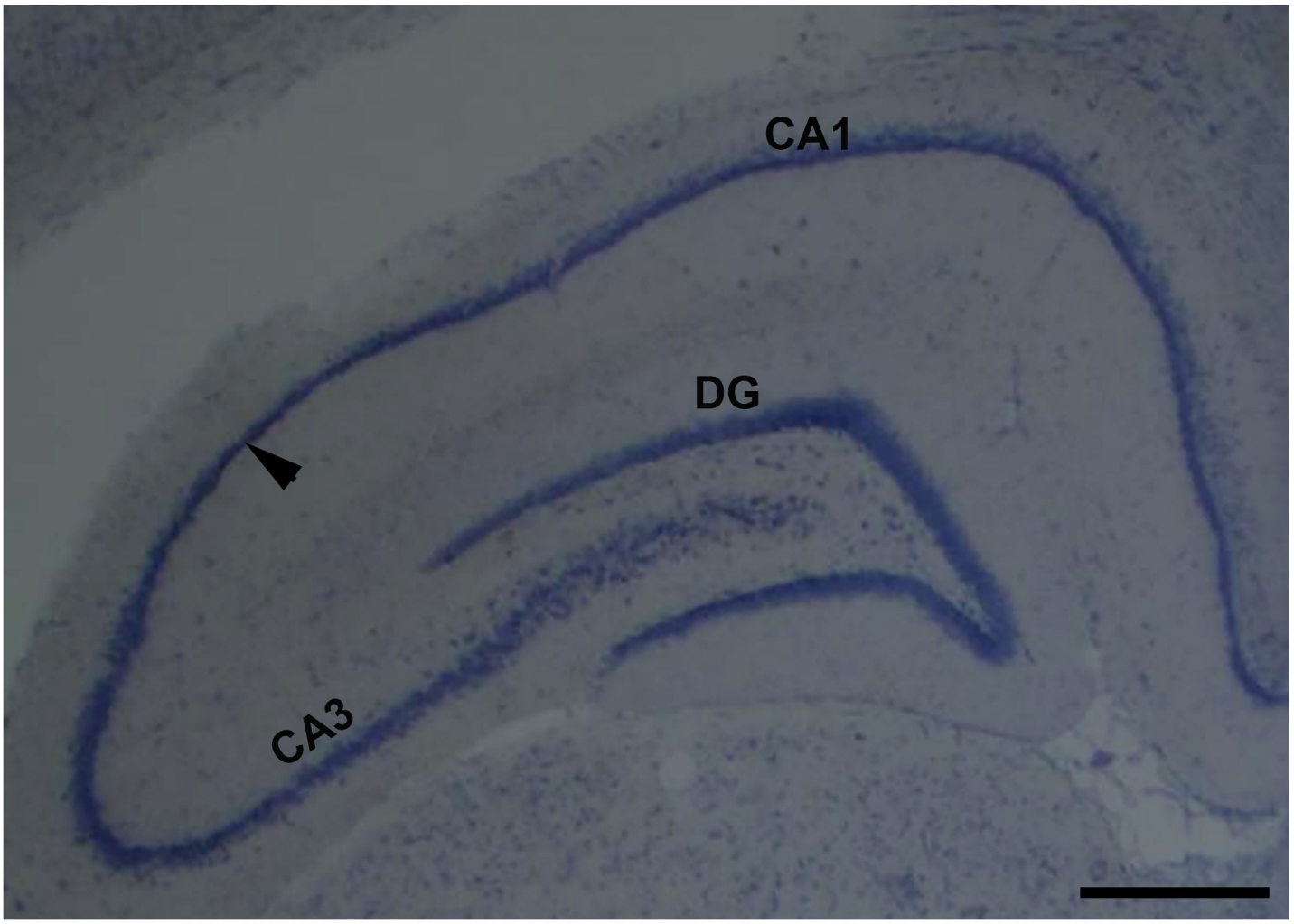
	OX-treated group			Vehicle treated group			p value
	Mean ± SD	CE	CV	Mean ± SD	CE	CV	
Total volume (mm^3)	31.84 ± 1.24[a]	0.02	0.03	36.95 ± 3.48[b]	0.02	0.09	0.01
Granular cell number (10^5)	7.36 ± 1.42	0.11	0.19	8.147 ± 2.84	0.11	0.34	0.69

Pyramidal cell number in CA1 (10^5)	3.521 ± 0.54	0.11	0.15	4.257 ± 0.59	0.1	0.13	0.19
Pyramidal cell number in CA3 (10^5)	1.989 ± 0.46	0.12	0.23	2.133 ± 0.22	0.12	0.1	0.33

Mean ± SD of hippocampal volume and neurons from one hemisphere (five animals in each group) estimates with CE (= SEM/mean; intera-animal coefficient of error) and CV (= SD/mean; observed inter-animal coefficient of variation). Different superscript letters in the same rows indicate a significant difference, $p < 0.05$.

Figure 1. Histological coronal section of the hippocampus (scale bar= 200 μm) with magnification of the CA1, CA3 and DG (scale bar= 500 μm). Arrowhead mark boundary between CA1 and CA3.

Figure 2. Stereological method for estimating hippocampal volume and neuronal number. (a): Example of a point grid on histological section of the hippocampus. The total number of points hitting the whole hippocampus was counted. (b): Example of a unbiased counting frame used for optical dissector method. The cells which their nucleolus came into focus within dissector 's height if they were completely inside the counting frame or touched the accepted lines (green lines) were counted. Here one neuron (asterisk) was counted (scale bar= 200 μm).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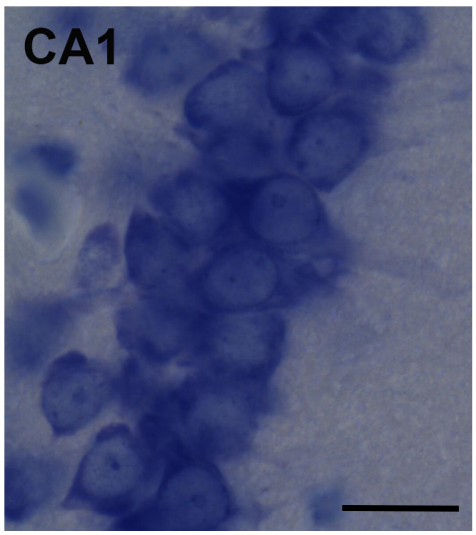
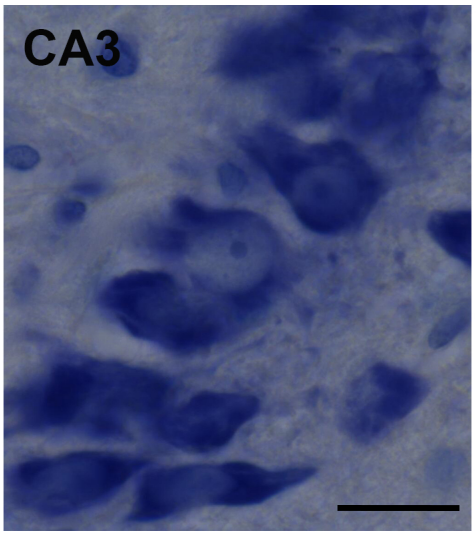
DG
CA3


CA1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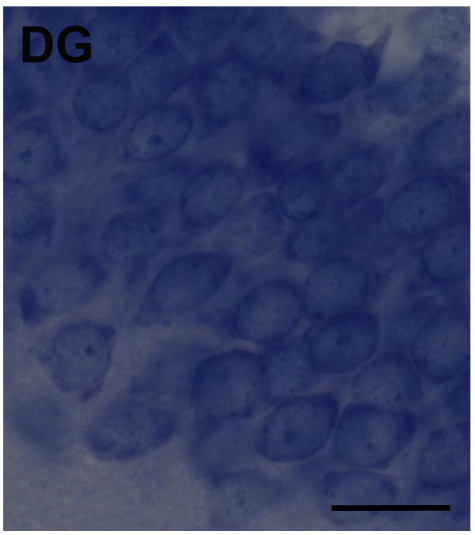
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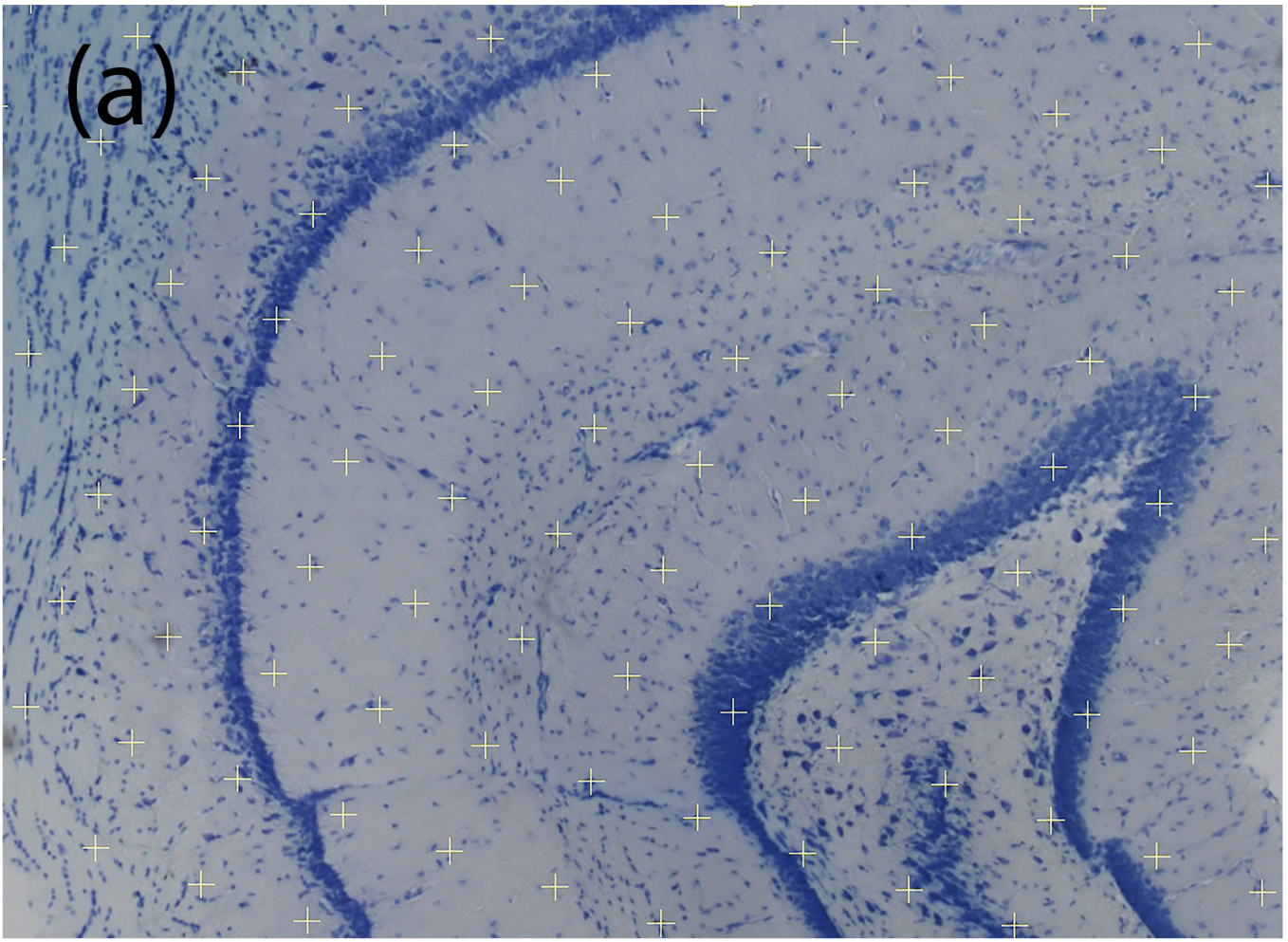
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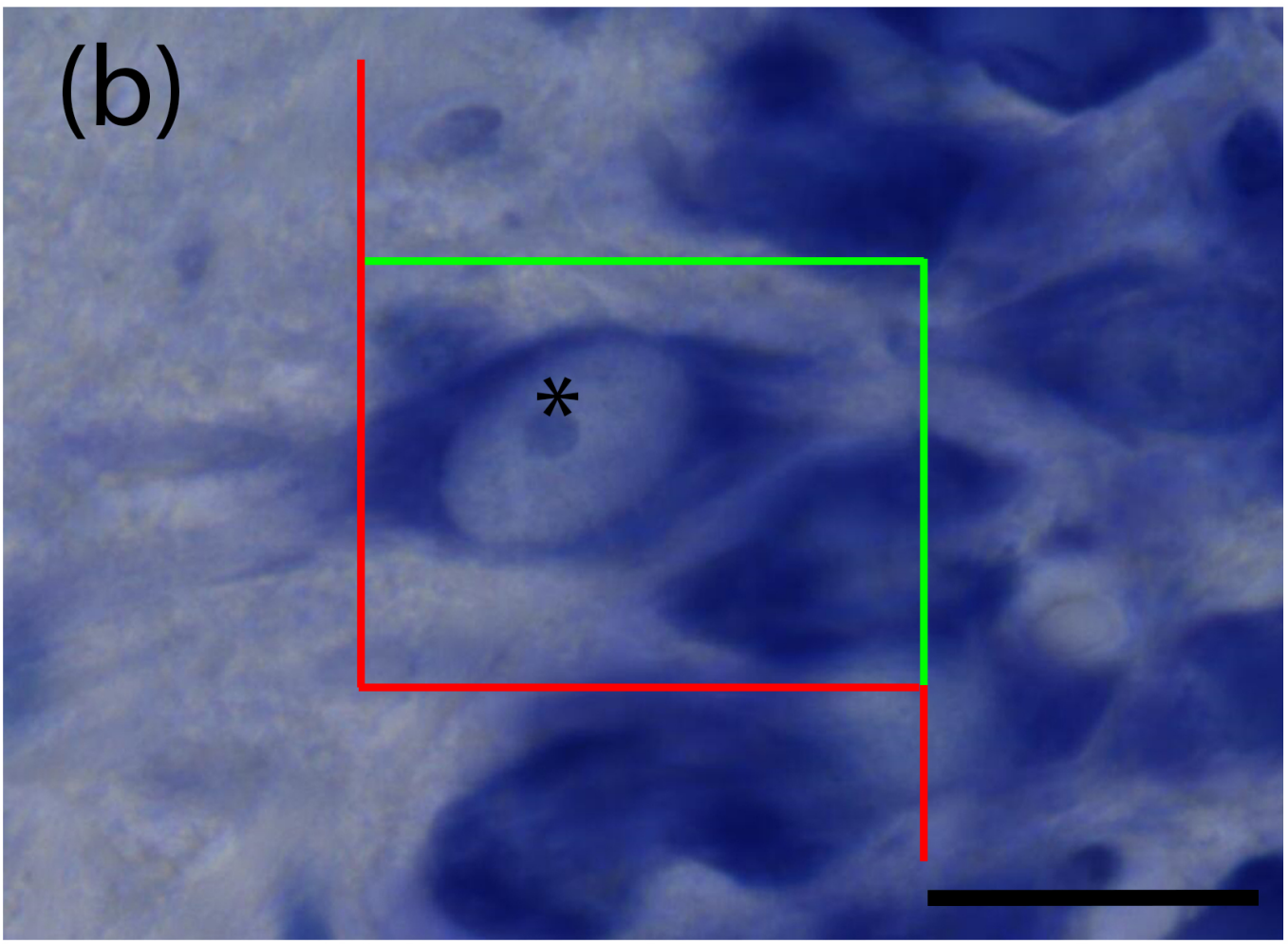
(b)
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